Mueller Hinton II Broth (Cation-Adjusted)
Mueller Hinton II Broth (Cation-Adjusted) with 2% Sodium Chloride

Intended Use

Mueller Hinton II Broth is intended for use in quantitative procedures for susceptibility testing of rapidly-growing aerobic and facultatively anaerobic bacteria isolated from clinical specimens. It is formulated to have a low thymine and thymidine content and is adjusted to the calcium and magnesium ion concentrations recommended in CLSI standard M7.[1]

Mueller Hinton II Broth with 2% Sodium Chloride (NaCl) is for testing methicillin-resistant strains of *Staphylococcus aureus* (MRSA).[1]

Summary and Explanation

The development of laboratory tests to determine the activity of antimicrobial agents has paralleled the development of these agents. In 1929, Fleming used a serial dilution technique to measure the lowest concentration of penicillin that prevented growth of a test organism in broth.[2] Ericsson and Sherris published an excellent review of the various methods for susceptibility testing and the relationship of dilution and diffusion methods.[3]

Rammelkamp and Maxon were among the earliest to use the tube dilution test to determine the *in vitro* antimicrobial susceptibility of bacteria isolated from clinical specimens.[4] The development of this test resulted from the need to know why some patients infected with *S. aureus* did not respond to penicillin therapy.

The tube dilution test (broth dilution) involves exposing bacteria to decreasing concentrations of antimicrobial agents in liquid media, usually by serial two-fold dilution. The mixture, consisting of microorganisms, nutrient medium and antimicrobial agent, is incubated at 35°C for 16-20 hours. The lowest concentration of antimicrobial agent at which no visible growth occurs is defined as the minimal inhibitory concentration (MIC).

The term "microdilution" appeared in the literature in 1970 to describe the minimal inhibitory concentration tests performed with volumes of 0.1 mL or less of antimicrobial solution.[5] Correlations between MIC values using microdilution and tube dilution methodologies have been reported to be between 85 and 96%.[6,7]

The qualitative disc diffusion antimicrobial susceptibility procedure has been standardized since 1966.[8] The rationale for an MIC susceptibility test rather than the disc diffusion test is that it gives quantitative information. It provides a relationship between the amount of antimicrobial agent required to inhibit the growth of an organism *in vitro* and the achievable concentrations in the blood, urine, cerebrospinal fluid or bile, under various dosage conditions. It has been suggested that in the treatment of systemic infections, the drug dosage should yield a peak concentration at the site of infection that is two to four times greater than the MIC value, while for urinary tract infections, a peak urine concentration of 10-20 times the MIC value should be achieved.[9] However, effective antimicrobial therapy also depends on many other factors.[10]

Cation-adjusted Mueller Hinton Broth is the medium usually used for dilution antimicrobial susceptibility tests. This medium is supplemented with calcium and magnesium salts to produce correct MICs with aminoglycosides and *Pseudomonas aeruginosa*.[1] However, this medium is not satisfactory for fastidious organisms such as *S. pneumoniae*. Cation-adjusted Mueller Hinton Broth supplemented with 2-5% lysed horse blood is the medium recommended for susceptibility testing of *S. pneumoniae*.[1]

Thornsberry and McDougal reported that adding 2% sodium chloride to cation-adjusted Mueller Hinton Broth improved the reliability of MIC tests using oxacillin for detecting methicillin-resistant *S. aureus* (MRSA).[11] In addition, they recommend the alternative direct inoculum standardization procedure (see "Procedure," step 2) and incubation of the inoculated MIC trays or tubes for a full 24 hours.[11]

Principles of the Procedure

Acid hydrolysate of casein and beef extract provide nutrients for growth of test organisms. These ingredients are selected for low thymine and thymidine content as determined by MIC values with *Enterococcus faecalis* and sulfamethoxazole-trimethoprim (SXT). Calcium and magnesium ion concentrations are adjusted to provide the amounts recommended by CLSI to give the correct MIC values with aminoglycosides and *P. aeruginosa*.[1] The pH has been adjusted to the specification in CLSI standard M7.

User Quality Control

Identity Specifications

BBL™ Mueller Hinton II Broth (Cation-Adjusted)

Dehydrated Appearance:	Fine, homogeneous, free of extraneous material.
Solution:	2.2% solution, soluble in purified water upon boiling. Solution is pale to light yellow to tan, clear to slightly hazy.
Prepared Appearance:	Pale to light yellow to tan, clear to slightly hazy.
Reaction of 2.2% Solution at 25°C:	pH 7.3 ± 0.1
Calcium:	20-25 mg/L
Magnesium:	10-12.5 mg/L

Cultural Response

BBL™ Mueller Hinton II Broth (Cation-Adjusted)

Prepare the medium per label directions. Inoculate with approximately 10^5 of the test organisms, dispense into an antimicrobial susceptibility test system and incubate at 35 ± 2°C for 16-20 hours.

ORGANISM	ATCC™	RESULT
Enterococcus faecalis	29212	Satisfactory MIC values
Escherichia coli	25922	Satisfactory MIC values
Pseudomonas aeruginosa	27853	Satisfactory MIC values
Staphylococcus aureus	29213	Satisfactory MIC values

MRSA cultures often consist of two populations, one that is susceptible and one that is resistant (so-called "occult resistant" strains). The methicillin-resistant population grows more slowly and prefers a high salt concentration as contained in Mueller Hinton II Broth with 2% NaCl. In addition, the lower pH of this medium (6.9) improves the stability of β-lactam antibiotics during storage of prepared MIC test tubes or trays.[12]

Antimicrobial agents are prepared in serial two-fold dilutions in Mueller Hinton II Broth and are inoculated with the test culture to give a final concentration of 5×10^5 CFU/mL. Following incubation at 35°C, the presence of turbidity indicates growth of the organism. The lowest concentration of antimicrobial agent showing no growth is the MIC of that organism for that agent. The interpretation as to whether the organism is susceptible, intermediate, or resistant in its response to the agent is made by comparing the MIC to those in the MIC interpretive standards in CLSI standard M7.[1,13]

Various factors have been identified as influencing broth dilution susceptibility tests. These include the medium, antimicrobial potency, inoculum concentration, pH, antimicrobial stability and mechanisms of resistance by the test organisms.[3,14,15]

Formula

BBL™ Mueller Hinton II Broth (Cation-Adjusted)

Approximate Formula* Per Liter		
Beef Extract	3.0	g
Acid Hydrolysate of Casein	17.5	g
Starch	1.5	g

**Adjusted and/or supplemented as required with appropriate salts to provide 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium and as additionally required to meet performance criteria.*

Directions for Preparation from Dehydrated Product

1. Suspend 22 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 116-121°C for 10 minutes. DO NOT OVERHEAT.
4. Test samples of the finished product for performance using stable, typical control cultures.

Procedure

Mueller Hinton II Broth (Cation-Adjusted) may be used for inoculum preparation for MIC tests and for preparation of antimicrobial dilutions for the microdilution or macrodilution procedure. Details for the preparation of antimicrobial agents are provided in reference 1.

1. Inoculum Standardization (for rapidly growing bacteria)
   a. Using aseptic technique, pick 3-5 isolated colonies of the same organism from an 18- to 24-hour **Trypticase**™ Soy Agar with 5% Sheep Blood (TSA II) plate and inoculate into 5 mL of Mueller Hinton II Broth.
   b. Incubate 2-6 hours at 35°C. Periodically check turbidity against the 0.5 McFarland turbidity standard.
      - If comparable, go to step 3, Inoculation of Antimicrobial Dilutions.
      - If too turbid, dilute aseptically with additional Mueller Hinton II Broth and repeat turbidity check. If turbidity is comparable to the standard, go to step 3, Inoculation of Antimicrobial Dilutions.
      - If not turbid enough, continue incubation. When turbidity is comparable to the standard, go to step 3, Inoculation of Antimicrobial Dilutions.

   Suspensions of test organisms must be used within 15 minutes of standardization.
2. Alternative Direct Inoculum Standardization (for rapidly growing bacteria and MRSA)
   A stationary phase culture may also be used. In this method, skip step number 1b and simply suspend enough colonies in the broth to equal the turbidity of the 0.5 McFarland standard. For MRSA, use Mueller Hinton II Broth with 2% NaCl. Suspensions of test organisms must be used within 15 minutes of standardization.
3. Inoculation of Antimicrobial Dilutions
   The amount of inoculum depends on the procedure used.[1] The standardized inoculum prepared above will contain approximately 1-2 × 10^8 CFU/mL. The final concentration

in a well (or tube) should be 5×10^5 CFU/mL (*not* CFU/tube or well).

a. Macrodilution (tube) method
If the volume of antimicrobial solution in the tube is 1 mL, dilute the standardized inoculum 1:100 in Mueller Hinton II Broth (0.1 mL to a 10-mL tube of broth). Add 1.0 mL of the adjusted inoculum to each tube containing an antimicrobial agent and 2.0 mL to a sterile empty tube for a growth control.

b. Microdilution method
In this method, the antimicrobial dilutions are made in sterile plastic trays with round or conical-shaped wells. The volume is either 0.05 or 0.1 mL in each well. If the volume in the well is 0.1 mL, dilute the inoculum 1:10 and add 0.005 mL of the inoculum per well, using a replicator. One well in each tray should contain 0.1 mL of broth without any antimicrobial agent (growth control well).
If a dropper (0.05 mL) is used for the inoculum and the volume of antimicrobial solution is 0.05 mL, this results in a 1:2 dilution. Therefore, dilute the inoculum 1:100 and add 0.05 mL to each well to obtain the final concentration of 5×10^5 CFU/mL (5×10^4 CFU/well). Add 0.05 mL of broth without any antimicrobial agent (growth control well). After the trays are inoculated, cover with tape or a tight-fitting lid to prevent evaporation.

4. Incubation
Incubate the tubes or trays (stacked no more than four high) at 35°C for 16-20 hours for Mueller Hinton II Broth and a full 24 hours for Mueller Hinton II Broth with 2% Sodium Chloride (MRSA). Do not use a CO_2 incubator. To prevent drying out, the trays should be covered with plastic tape, a tight fitting lid, or placed in a plastic bag.[1]
Control cultures should be included each time a susceptibility test is performed or weekly if satisfactory performance can be documented according to the CLSI standard.[1] The correct quality control MIC ranges will be found in M100 (M7).

Expected Results

The minimal inhibitory concentration (MIC) of an antimicrobial agent for a specific organism is the lowest concentration which will inhibit the growth of the organism. Growth is indicated by turbidity or sediment. Some microorganisms when tested against trimethoprim/sulfamethoxazole or sulfonamides alone do not always give clear-cut end points. In the case of doubling dilutions of trimethoprim/sulfamethoxazole, there may be a "trailing" of growth. Such a pattern typically shows an obvious reduction in the amount of growth and, then, either small pellets (usually less than 1 mm in diameter) in the rest of the wells, or an obvious reduction in the amount of growth and then a slight but detectable graduation in the size of the pellets. In these cases, the MIC end point should be identified as the lowest concentration of antimicrobial agent beyond which there is no further reduction in the size of the pellet or amount of turbidity.

An organism may be susceptible, intermediate or resistant for a given antimicrobial agent depending on the MIC value. Interpretive standards for MIC values with various drugs may be found in CLSI document M100 (M7)[1] or may be obtained from the drug manufacturer.

NOTE: Informational supplements to CLSI Document M7, containing revised tables of antimicrobial agents and interpretive standards are published periodically. The latest tables should be consulted for current recommendations. The complete standard and informational supplements can be ordered from the Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898. Telephone: (610) 688-1100.

Refer to other texts for additional information on antimicrobial susceptibility testing.[16,17]

References

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Availability

BBL™ Mueller Hinton II Broth (Cation-Adjusted)

BS12 CLSI CMPH2 MCM9

Cat. No.	212322	Dehydrated – 500 g
	297701	Prepared Tubes, 5 mL (K Tubes) – Pkg. of 10
	298268	Prepared Tubes, 5 mL (K Tubes) – Ctn. of 100
	297310	Prepared Bottle (pH 7.3) – 250 mL
	297963	Prepared Bottle – 400 mL

BBL™ Mueller Hinton II Broth (Cation-Adjusted) with 2% Sodium Chloride

CLSI

Cat. No.	297311	Prepared Bottle (pH 6.9) – 250 mL